Langerhans Cells in S-phase in Normal Skin Detected by Simultaneous Analysis of Cell Surface Antigen and BrdU Incorporation

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We report on a double immunofluorescence staining for the detection of the Langerhans Cell (LC) population in S-phase. After trypsinization, the epidermal cell suspensions were enriched for LC and exposed to 10 μM BrdU for 2 h. Studies of BrdU labeled cells included the determination of their cell surface phenotype. Both membrane labeling and incorporated BrdU as revealed using anti-BrdU demonstrated the presence of LC in S-phase. We observed 6% of the epidermal LC in S-phase. This is a proof that LC are able to proliferate in the epidermal microenvironment. This technique, besides being rapid and free of radioactivity, allows the cytokinetic study of phenotypically defined LC in heterogeneous epidermal cell populations. *J Invest Dermatol 91:603–605, 1988*

The Langerhans cells (LC) are migratory bone marrow-derived dendritic cells with a specific intracytoplasmic organelle, the Birbeck granule. They express HLA-DR (MHC-class II) molecule, and thereby, antigen-presenting cells represent the major accessory cells of Skin Associated Lymphoid Tissue (SALT) [1–3]. The T6 antigen, a common antigen expressed by LC and the cortical thymocytes, is a frequently used surface marker for the study of epidermal LC in human skin [4–6]. LC comprise 2%–4% of all epidermal cells [1].

Previous studies based on [3H] Thymidine incorporation [7,8] into the nuclei reported LC in S-phase but, in these studies, the identification of LC was uncertain because they were not identified by immunologic markers. More recently, immunocytochemical studies have shown that the number of LC was increased in certain pathologic conditions [9,10], but in these cases the presence of cells in S-phase or mitosis was not proved. Thus far, only one study has been published where the authors evaluated the LC replication potential in human skin grafted on nude mice. Mice bearing two-month old human skin grafts were injected with BrdU and the incorporated BrdU and LC were revealed at the same time on skin sections using Fluorescein isothiocyanate (FITC) conjugated anti-BrdU and OKT6 monoclonal antibodies (Mab) [11].

We report here the detection of replicating LC performed directly on epidermal cell suspensions from normal human skin. We define BrdU incorporation staining and fixation conditions enabling the simultaneous detection of nuclear BrdU and surface antigen on epidermal LC in suspension.

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Abbreviations:

BrdU: Bromodeoxyuridine
LC: Langerhans Cells
MEM: Minimal Essential Medium
PBS: Phosphate Buffered Saline
FITC: Fluorescein isothiocyanate

MATERIALS AND METHODS

Epidermal LC Cell suspensions were obtained by trypsinization of human normal skin specimens taken during plastic surgery, as described in [12]. LC enrichment was obtained by Ficoll-Hypaque (Pharmacia, France) sedimentation [12].

In Vitro BrdU Incorporation Enriched-LC suspensions were incubated for 2 h at 37°C in MEM containing 10% Fetal Calf Serum (FCS) and BrdU at a final concentration of 10 μM. After two washes in MEM-5%FCS, the cells were resuspended at 1×10^6 cells/ml.

Simultaneous Cell Surface and Nuclear Staining The cells were stained for specific membrane antigen using the Mab OKT6 (Ortho, Raritan, NJ) followed by biotinylated $F(ab')_2$ goat anti-mouse IgG (Immunotech, Luminy, France) for 30 min at 4°C. The antibody was revealed by rhodamine-conjugated avidin (Vector, Burlingame, CA) for an additional 30 min at 4°C. The pellet was washed twice in Phosphate buffered saline (PBS)—1% glucose and fixed in 70% ethanol for 30 min at 4°C. The cells were then treated by 2N hydrochloric acid (HCl) for 30 min at 20°C. The acid was neutralized in 0.1 M Borax (pH 8.5) for 5 min and the cells were washed in PBS.

The incorporated BrdU was revealed by a monoclonal antibody anti-BrdU (Becton Dickinson, Mountain View, CA) at a 1/10 dilution in PBS containing 0.5% Tween 20 and 10% Bovin Serum Albumin (BSA) for 30 min at 20°C. After two washes in PBS-10%BSA, the intranuclear label was revealed by FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (Zymed, San Francisco, CA) for 30 min at 20°C. After two washes in PBS-10%BSA, cells were observed under a Zeiss Orthoplan microscope with selective excitation filters for fluorescein and rhodamine. Controls were performed in the absence of either OKT6 staining or anti-BrdU treatment.

RESULTS

In epidermal cell suspensions fractioned by Ficoll-Hypaque sedimentation, LC accounted for approximately 20%. LC-enriched cells were then distributed into 10^6 cells per assay.

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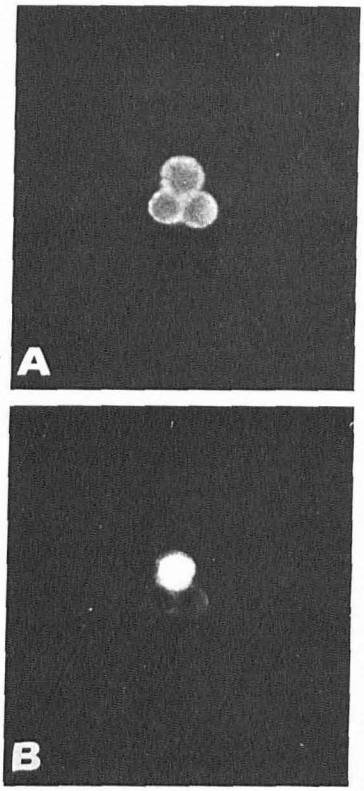

Figure 1. Langerhans cells double labeled with OKT6 revealed in *A* by biotin-avidin rhodamine and with anti-BrdU revealed in *B* by FITC. In this field one LC (OKT6 +) has entered the S phase as revealed by the nuclear staining with the anti-BrdU (× 500).

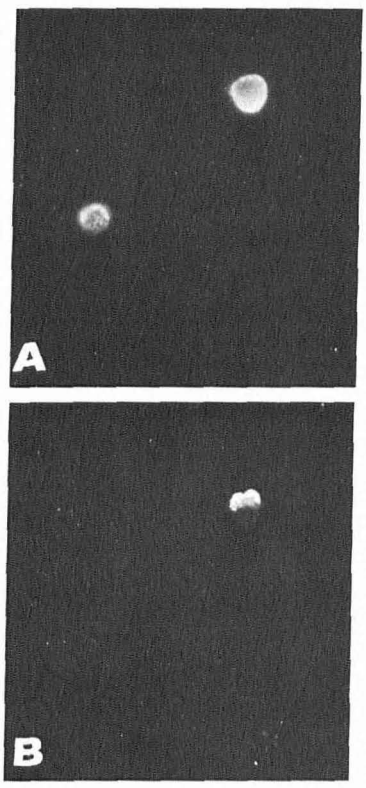

Figure 2. A different LC suspension treated as in Fig 1. Note the reniform shape of the nucleus. (× 500).

Table I. Simultaneous Analysis of Cell Surface Antigen and BrdU Incorporation of Epidermal LC.

	Skin samples[a]					
	1	2	3	4	5	6
BrdU+ T6+[b] (percentage)	6.0	6.1	5.8	5.5	7.5	6.0

[a] The assay was performed on six different skin specimens.
[b] Mean percentage on 1000 counted cells in randomly chosen fields for each skin sample.

The cells positive for T6 antigen exhibited a bright red fluorescence confined to the cell surface membrane and the labeling had a fine granular aspect (Fig 1*a* and 2*a*).

The nuclei positive for BrdU showed a bright green fluorescence (Fig 1*b*). A reniform shape of the nucleus (known to be characteristic of LC) could be observed (Fig 2*b*). No labeling was observed in the cytoplasm.

BrdU incorporation ranging from 30 min to 6 h were tested. It was found that constant results were obtained after an incorporating time of 2 h or more. Two hours was then chosen as the time of incorporation in our study.

Six different skin specimens were tested and a mean of 6% (5.5 to 7.5%) of the T6 positive cells were also found to be BrdU positive (Table I). This is a mean percentge on 1000 counted cells in randomly chosen fields for each skin sample. Some BrdU positive and T6 negative cells were observed. These cells corresponded to basal keratinocytes. Controls were negative indicating the specificity of the reaction.

DISCUSSION

In previous studies, the proliferation activity of the LC was established on skin sections by tritiated thymidine incorporation [7,8]. Though the labeling indices observed were low (0.125%), these findings showed that LC can proliferate in normal skin. The low percentage (2% – 4%) in normal skin and the low labeling index of LC represent factors limiting the study of their cell cycle.

Recently, a method for the evaluation of the LC potential replication in epidermis was reported by Czernielewski et al [11]. They grafted normal human skin on nude mice and after 2 months, grafts were used to estimate the LC in S-phase. BrdU was injected in mice and then skin sections were taken at various times. Under these conditions, the LC in S-phase was estimated to be 4.9% in normal skin. Though this method appears reliable, it is not easy to perform because it requires the technology of skin grafting on mice and is also time consuming because it takes 2 months before any valid result can be obtained.

We have developed an easy and short method that can be applied to epidermal cells in suspension. By an in vitro test performed on a LC-enriched epidermal populations, we found 6% of LC in S-phase. These results are consistent with those obtained by Czernielewski et al [11]. The method, described here, is rapid, specific, and does not involve radioactivity. Since LC account for 2% to 4% in normal epidermis, enrichment was performed to facilitate the study. The number of LC was thereby increased up to 20%, but this enrichment does not allow subpopulations of LC to be selected [12]. This enrichment provides possibilities of testing the potential replication of LC from small specimens of skin. The conditions of enrichment and a three-step monoclonal antibody biotin-avidin rhodamine technique were necessary to detect a small cell population. The specificity of the intranuclear BrdU and membrane antigen labelings was very well preserved. Background staining was absent.

This method offers the possibility to obtain a number of rapid and valuable insights into the cell kinetics of LC in normal as well as in pathologic situations. Several studies have shown increased mitotic activity of LC after physical damage (UV light irradiation [1] or tape stripping [13] in burns [14]) or during pathologic processes including histiocytosis X [1], mycosis fungoides [9], squamous cell carci-

noma, and seborrheic keratosis [10]. These studies using autoradiography and cytochemistry suffer from the uncertainty of LC identification at the light microscope level.

This in vitro test represents a specific, sensitive, and rapid method to establish the proliferative activity of LC. It can be used to investigate the influence of chemical products on LC kinetics in normal skin.

Finally, our data demonstrate that LC are cycling and that they are a stable, self-reproducing cell population in normal human epidermis. The presence of LC in S and G2/M phases was also demonstrated using flow cytometry-DNA measurement [15], but the contaminating keratinocytes could influence the results. The method described here also offers the possibility of a more precise and reliable analysis of the cell cycle of LC using flow cytometry.

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